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(54) **METHOD FOR INHIBITING CONIDIAL GERMINATION AND MYCELIAL GROWTH OF FUNGI SYMBIOTICALLY ASSOCIATED WITH BARK BEETLES**

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***A61K 31/425*** (2006.01)
***A01N 43/78*** (2006.01)

(52) **U.S. Cl.**
CPC ..... ***A01N 43/78*** (2013.01)

(58) **Field of Classification Search**
CPC ..... A61K 31/425; A01N 43/78
See application file for complete search history.

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(57) **ABSTRACT**

A method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth. A method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth. The anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid. The species of the fungal cell is selected from a group that has an obligate symbiosis with the mountain pine beetle (*Dendroctonus ponderosae*) and the western pine beetle (*Dendroctonus bervicomis*).

**8 Claims, 6 Drawing Sheets**

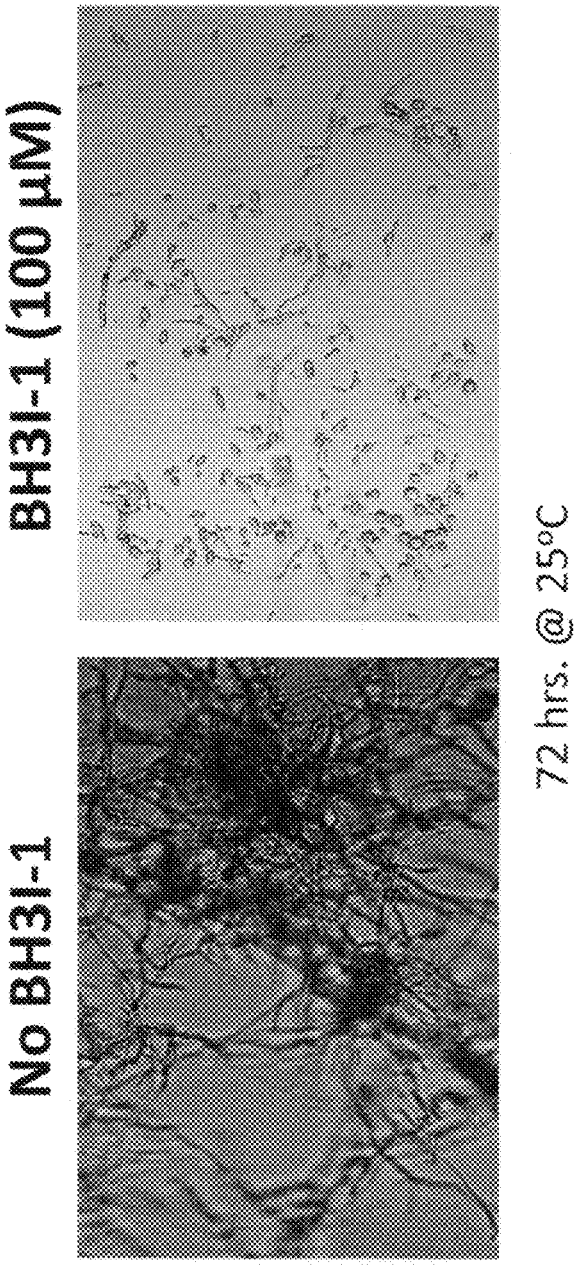
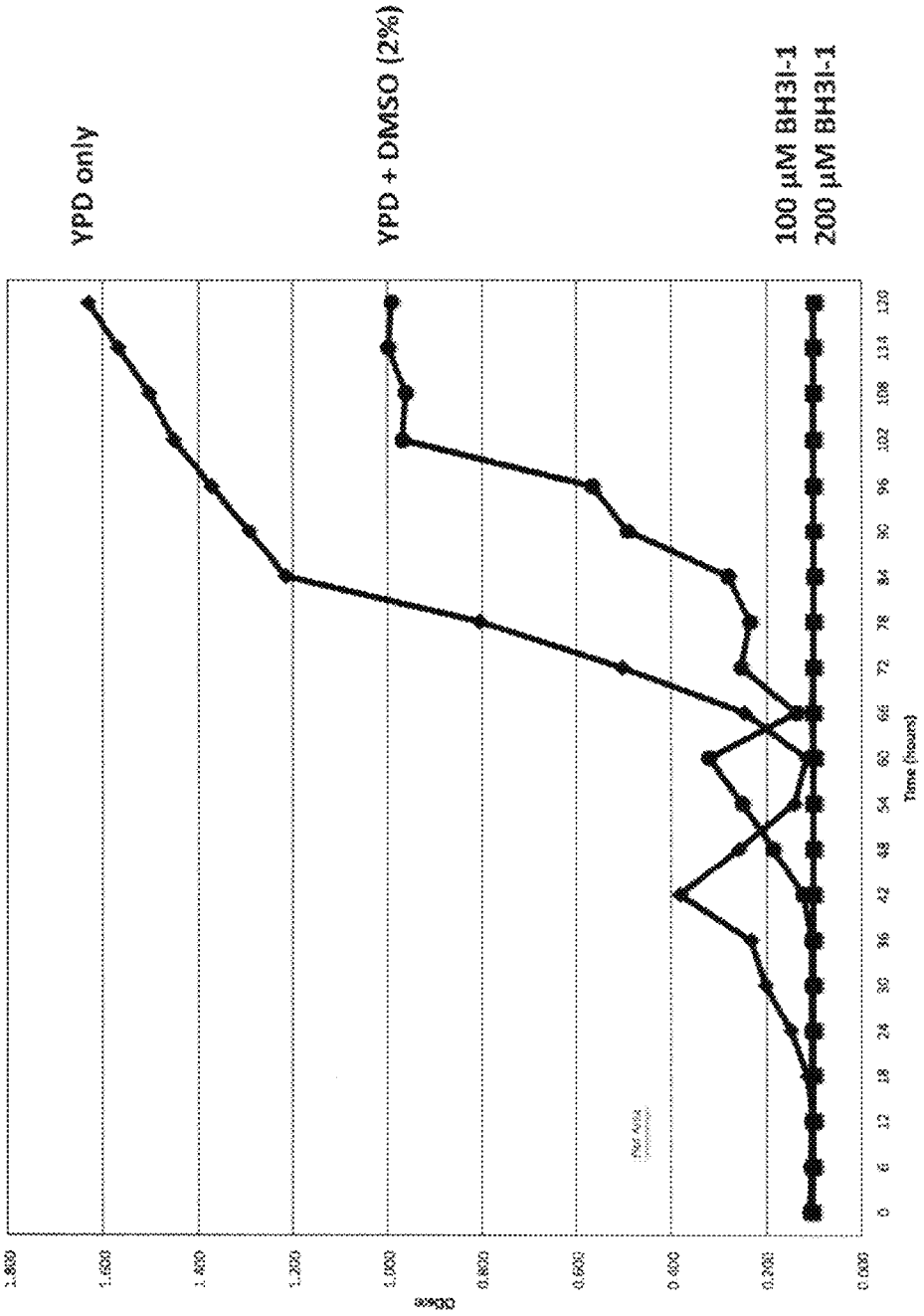



Figure 1

Figure 2


Grossmannia clavigera
YPD only
YPD + DMSO (2%)
100 µM BH3I-1
200 µM BH3I-1
OD600
Time (hours)

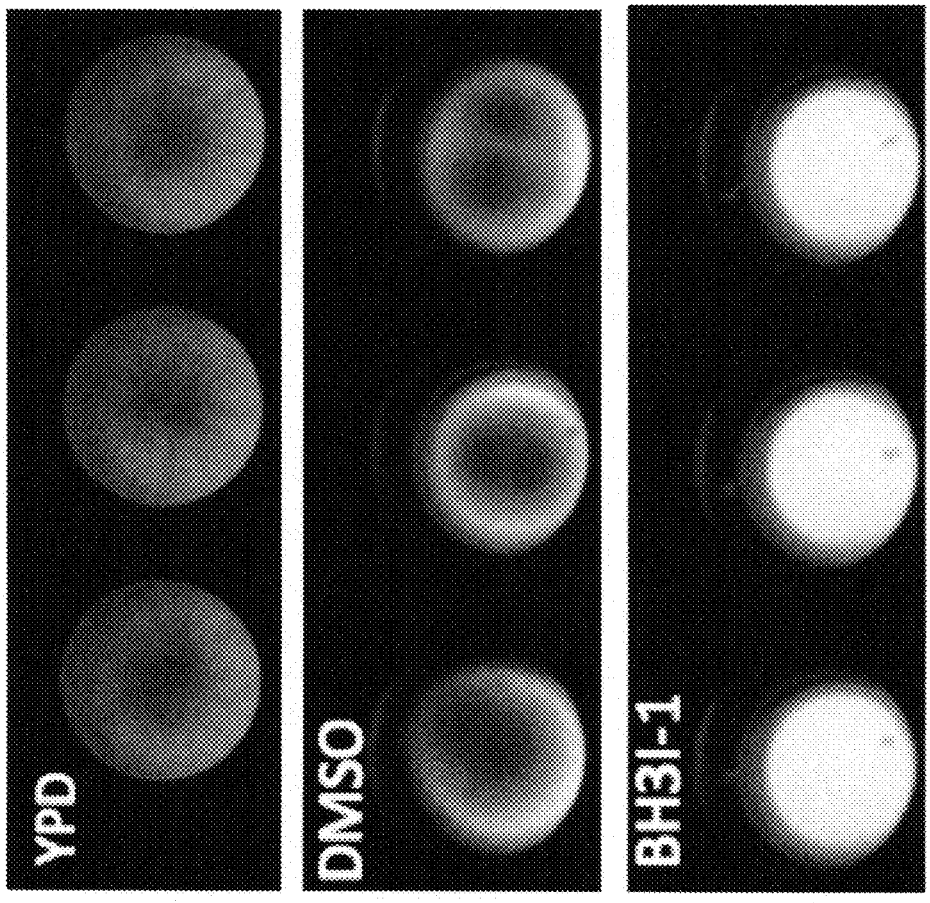



Figure 3

Figure 4

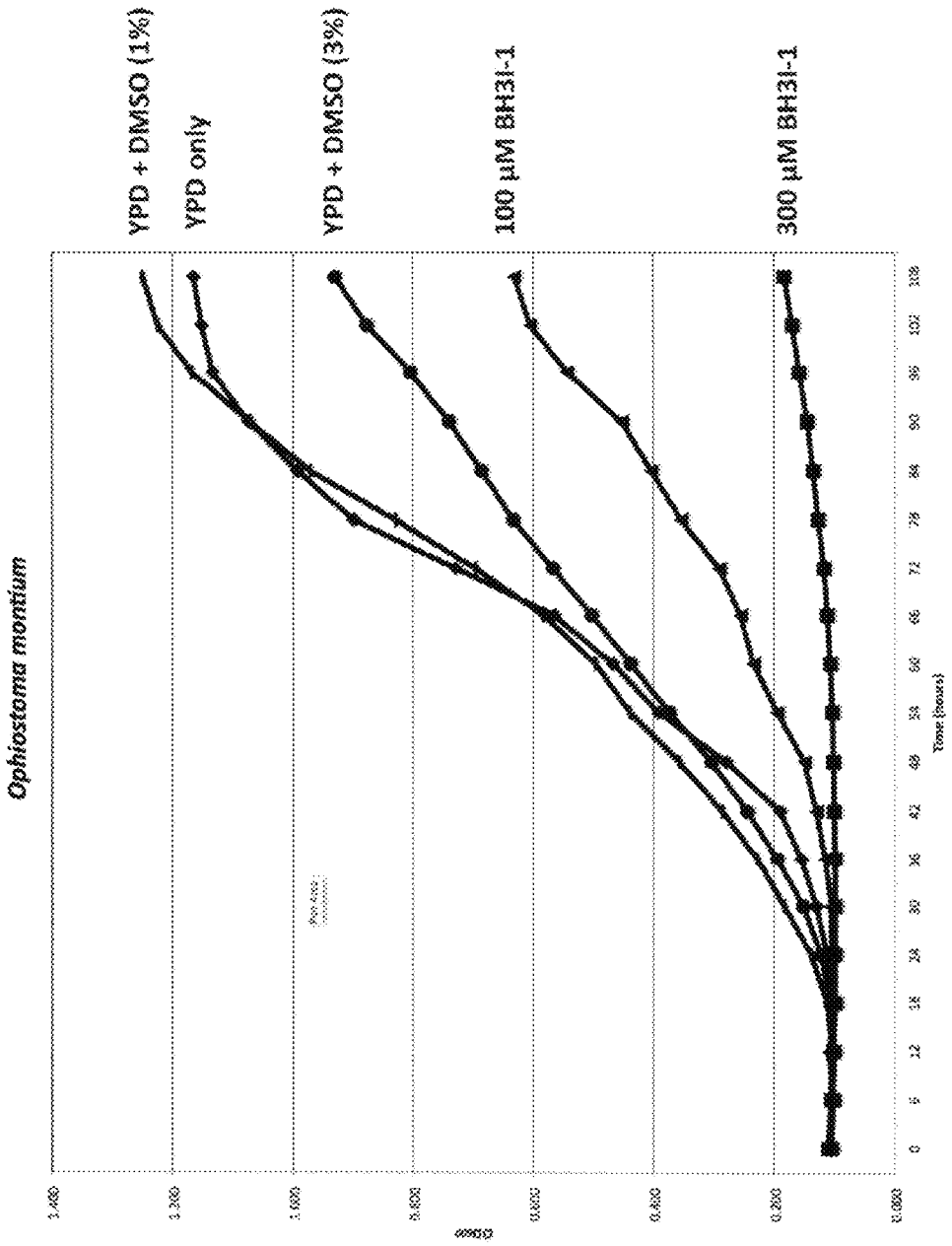
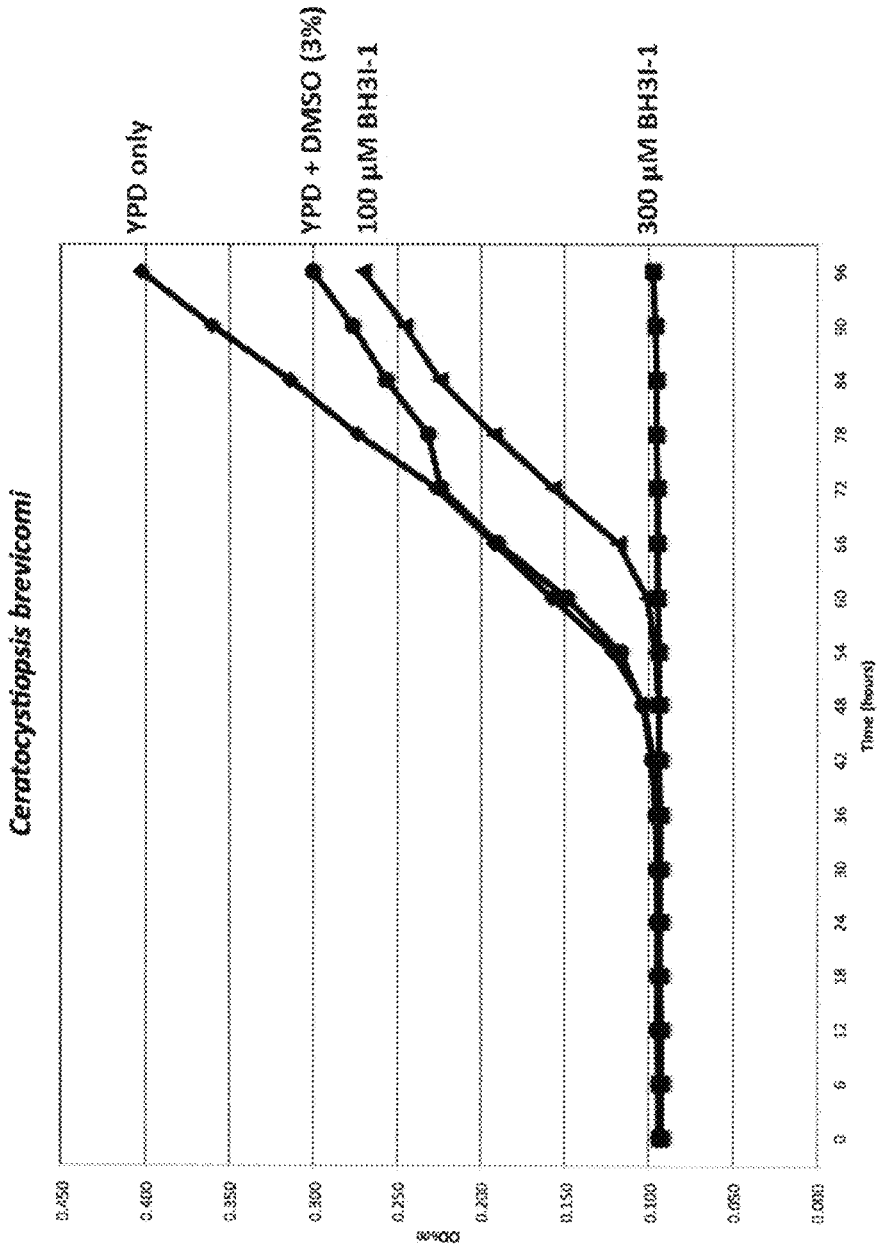

Ophiostoma montium
YPD + DMSO (1%)
YPD only
YPD + DMSO (3%)
100 µM BH3I-1
300 µM BH3I-1
OD600
Time (hours)

Figure 5


Ceratocystiopsis brevicomi
YPD only
YPD + DMSO (3%)
100 µM BH3I-1
300 µM BH3I-1
OD600
Time (hours)
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84
90
96

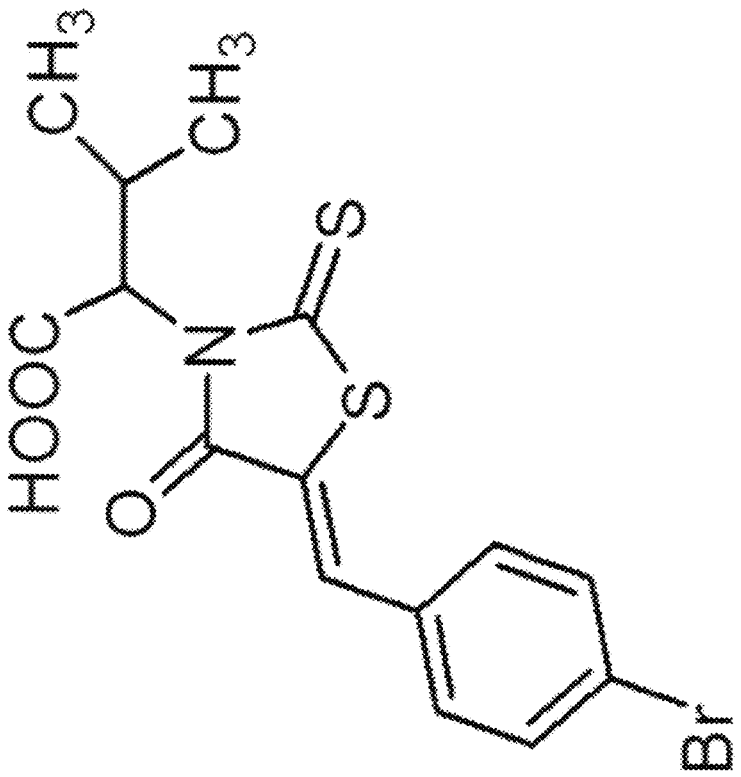



Figure 6

# METHOD FOR INHIBITING CONIDIAL GERMINATION AND MYCELIAL GROWTH OF FUNGI SYMBIOTICALLY ASSOCIATED WITH BARK BEETLES

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Award No. PG15-66120-01 to Montana State University, Sub-Award No. G119-15-W4747 to Montana State Univeristy-Billings, awarded by the National Science Foundation. The government has certain rights in the invention.

## BACKGROUND Of THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of molecular biology, and more specifically, to a method for inhibiting conidial germination and mycelial growth of fungi that live symbiotically with various species of bark beetles.

2. Description of the Related Art

The present invention addresses the need to control infestations of coniferous trees by different species of bark beetles. Bark beetles, such as the mountain pine beetle *Dendroctonus ponderosae* and the western pine beetle *Dendroctonus brevicomis*, are responsible for killing large numbers of coniferous trees over vast areas of western North America. Indeed, beetle outbreaks are the leading cause of pine tree mortality in North America, and since the outbreak began in the late 1990s, approximately 45 million acres of pine trees have been killed in United States alone.

Bark beetles colonize pine trees as a natural part of their life cycle. During mid-summer, so-called "pioneer" beetles initiate tree colonization by boring through the bark and releasing pheromones that attract additional male and female beetles to a tree. The congregating beetles then mate, excavate an egg chamber within the phloem layer, lay eggs and die. After hatching, the larval progeny develop within the tree (carving out a system of tunnels as they feed on the sapwood) and emerge the following summer as adults. Many bark beetles colonize weak or dying trees; however, in the current outbreak, mountain pine beetles are colonizing and killing healthy trees.

Most species of bark beetles are host to a variety of fungal species from the genera *Grosmannia, Ophiostoma, Ceratocystiopsis* and *Ceratocystis* (1). Many of these fungal species have an obligate symbiotic relationship with bark beetles; that is, the fungi are required for the survival of the host bark beetle, most likely by providing access to tree nutrients (2,3,4). The beetles carry their symbiotic fungi as conidia in mycangia, specialized structures located on their exoskeleton. During colonization of a pine tree, the bark beetles passively introduce fungal conidia to the tree interior. Through conidial germination and mycelial growth, the fungi are able to invade phloem layer and sometimes also the xylem of the tree, discoloring the wood and disrupting water flow in the tree. It is not clear whether the fungi have a direct causal role in tree death (through mycelial invasion of the wood, for example) or whether they contribute indirectly to tree death through their symbiotic relationship with the beetles. In any case, the chemical inhibition of fungal growth—that is, the inhibition of conidial germination and/or mycelial growth—may provide a novel means of managing, controlling or limiting bark beetle infestations of pine trees.



The present invention is based on the unexpected discovery that the small molecule 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid (hereafter referred to as "BH3I-1") inhibits the conidial germination and mycelial growth of fungal species that have an obligate symbiotic relationship with the mountain pine beetle (*Dendroctonus ponderosae*) and the western pine beetle (*Dendroctonus bervicomis*). The fungal species inhibited by BH3I-1 include *Grosmannia clavigera* (symbiont of mountain pine beetle), *Ophiostoma montium* (symbiont of mountain pine beetle) and *Ceratocystiopsis brevicomi* (symbiont of western pine beetle).

The inventors do not think the inhibitory activity of BH3I-1 against bark beetle fungal symbionts is an obvious discovery in light of prior art. The inventors previously found that BH3I-1 inhibits morphogenesis (i.e., the yeast-to-filamentous growth transition) in *Candida albicans* (5). *C. albicans* and the bark beetle fungal symbionts share only a distant evolutionary history. Comparative genomic analyses have placed *C. albicans* and *G. clavigera* (the mountain pine beetle symbiont) in different taxonomic subphyla with a most recent common ancestor of approximately 350 million years ago (6,7). Because it has been shown that antifungal susceptibility can vary widely even among species of the same genus (8), there is no basis to expect that an inhibitory molecule will be active on such evolutionarily divergent fungi as *C. albicans* and the bark beetle fungal symbionts.

## BRIEF SUMMARY OF THE INVENTION

The present invention is a method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Grosmannia clavigera*. In an alternate embodiment, the present invention is a method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ophiostoma montium*. In another alternate embodiment, the present invention is a method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ceratocystiopsis brevicomi.*

The present invention is a method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Grosmannia clavigera*. In an alternate embodiment, the present invention is a method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small

molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ophiostoma montium*. In another alternate embodiment, the present invention is a method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ceratocystiopsis brevicomi*.

The present invention is a method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is selected from a group that has an obligate symbiosis with the mountain pine beetle (*Dendroctonus ponderosae*) and the western pine beetle (*Dendroctonus bervicomis*). The present invention is also a method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is selected from a group that has an obligate symbiosis with the mountain pine beetle (*Dendroctonus ponderosae*) and the western pine beetle (*Dendroctonus bervicomis*).

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows *G. clavigera* grown in YNB (yeast nitrogen base) medium without BH3I-1 (left panel) and with 100 μM BH3I-1 (right panel) for 48 hours at 25° C. The number of cells inoculated is the same for each condition.

FIG. **2** shows growth curves for *G. clavigera* over 120 hours at 25° C. in YPD medium only, YPD plus DMSO and YPD with 100 μM and 200 μM BH3I-1.

FIG. **3** shows photographs of triplicate wells of *G. clavigera* after 120 hours of incubation at 25° C. in YPD only, YPD plus DMSO and YPD plus 100 μM BH3I-1.

FIG. **4** shows growth curves for *O. montium* over 108 hours at 25° C. in YPD medium only, YPD plus DMSO and YPD with 100 μM and 300 μM BH3I-1.

FIG. **5** shows growth curves for *C. brevicomi* over 96 hours at 25° C. in YPD medium only, YPD plus DMSO and YPD with 100 μM and 300 μM BH3I-1.

FIG. **6** is a schematic representation of the chemical structure of BH3I-1.

## DETAILED DESCRIPTION OF INVENTION

### A. Overview

Currently very few options exist for controlling or limiting bark beetle infestations of coniferous trees. Chemical pesticides and beetle pheromone treatments are expensive and mostly ineffective. The present invention is based on the discovery of anti-fungal properties of the small molecule BH3I-1 against *G. clavigera, O. montium* and *C. brevicomis*, obligate symbionts of the mountain pine beetle and western pine beetle. The invention provides a method for managing, controlling and/or limiting bark beetle infestations of coniferous trees through inhibition of conidial germination and mycelial growth of the fungi symbiotically associated with bark beetles.

### B. Anti-fungal Properties of BH3I-1

The inventors have discovered that the small molecule BH3I-1 is effective for inhibiting conidial germination and mycelial growth of of *G. clavigera, O. montium* and *C. brevicomis*.

1. Assays for Inhibition of Conidial Germination and Mycelial Growth of Fungi.

A simple in vitro assay system was designed to detect inhibition of conidial germination and mycelial growth of *G. clavigera, O. montium* and *C. brevicomis*. Fungi are first grown in liquid medium at 25° C. for 48 hours hours, and conidia are harvested by filtration through cheese cloth. The conidia are then transferred to the wells of an optical microplate containing (i) growth medium and (ii) varying concentrations of BH3I-1 (typically 0, 50, 100, 200 and 300 μM BH3I-1). The microplates are incubated for up to 120 hrs at 25° C. Germination and mycelial growth are monitored visually with a Nikon TE200 inverted microscope and specrophotometrically in a Biotek Synergy H4 plate reader. For the spectrophotometric analysis, Optical Density 600 ($OD_{600}$) readings are taken every six hours over the course of the experiment.

2. Strains, Media and Chemicals.

Strains of *G. clavigera, O. montium* and *C. brevicomis* were obtained from Dr. Diana Six at the University of Montana and maintained on YPD plates (see below) at room temperarture.

YPD medium (1% Yeast Extract, 2% Peptone and 2% Dextrose), a standard fungal growth medium, is used for these experiments. YPD plates included 1.5% agar.

BH3I-1 was dissolved in dimethyl sulfoxide (DMSO) as a 5 mM stock and diluted directly into 100 μl of the appropriate YPD medium at 50 μM, 100 μM, 200 μM and 300 μM final concentration. The control condition (no BH3I-1) included medium plus DMSO to a concentration equivalent to that of the BH3I-1-containing wells.

3. Results.

In the presence of 100 μM BH3I-1, approximately 80% of *G. clavigera* conidial cells failed to germinate. For those that did germinate, mycelia grew for a few hours and then stopped (FIG. **1**). Growth inhibition was maintained for 120 hours (FIGS. **2** and **3**). In contrast, without BH3I-1, *G. clavigera* conidia germinated and mycelial growth continued to confluence (FIGS. **1**, **2** and **3**). BH3I-1 also inhibited conidial germination and mycelial growth in *O. montium* and *C. brevicomi* (FIGS. **4** and **5**).

### C. Structure of 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid (BH3I-1)

The anti-fungal morphogenesis small molecule BH3I-1 has the chemical structure shown in FIG. **6**.

According to some aspects of the invention, the BH3I-1 molecule used in the methods for inhibiting conidial germination and mycelial growth of fungi may be used as a substantially isomerically-pure compound or as a mixture of isomers. Preferably, isomerically-pure compounds are used. Isomerically-pure, as used herein, means that one isomer will be present in an amount ranging from 51 to 100%, but

not with respect to other impurities or other compounds that may be present. The term "isomer," as used herein, may refer to an E or Z isomer, an R or S isomer, an enantiomer or a diastereomer.

D. Practical Applications

BH3I-1 is useful for a variety of in vitro and in situ uses. In one application of the present invention, a fungal cell is contacted with BH3I-1 in an amount effective to reduce or inhibit conidial germination and/or mycelial growth. It is intended that the fungal cell is contacted either in vitro or in situ, whereby in situ includes contacting a fungal cell on the surface of or within a bark beetle. One of ordinary skill in the art would understand "contacting" to encompass putting a fungal cell into contact with BH3I-1, for example, in a culture plate or flask, whereby the fungal cell is placed into media containing BH3I-1. Further "contacting" would be understood by one of ordinary skill in the art to mean adding BH3I-1 to a fungal cell or population of fungal cells on the surface of or within a bark beetle.

As used herein, the term "fungal cell" is intended to encompass any cell originating from a fungal species or fungus. As used herein, the term "fungus" includes molds, yeast and pathogenic yeast. A fungus includes, but is not limited to, *Grosmannia* (for example, *Grosmannia clavigera*), *Ophiostoma* (for example, *Ophiostoma montium, Ophiostoma ips*) and *Ceratocystiopsis* (for example, *Ceratocystiopsis brevicomi*).

Although the invention has been described in connection with specific embodiments and applications thereof, the invention is capable of further modifications and/or applications, and this application is intended to cover any and all variations, uses, or adaptations of the invention that fall within the scope of the invention as described herein. The appended claims are therefore intended to cover all such variations, uses and adaptations as fall within the true spirit and scope of the invention.

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8. Schmalreck et al. (2014) Phylogenetic relationships matter: antifungal susceptibility among clinically relevant yeasts. Antimicrobial Agents and Chemotherapy. 58(3): 1575-1585.

We claim:

**1**. A method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Grosmannia clavigera*.

**2**. A method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ophiostoma montium*.

**3**. A method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ceratocystiopsis brevicomi*.

**4**. A method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Grosmannia clavigera*.

**5**. A method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ophiostoma montium*.

**6**. A method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is *Ceratocystiopsis brevicomi*.

**7**. A method for controlling conidial germination and mycelial growth in fungi comprising contacting a fungal cell with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth, wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is selected from a group that has an obligate symbiosis with the mountain pine beetle (*Dendroctonus ponderosae*) and the western pine beetle (*Dendroctonus bervicomis*).

**8**. A method for controlling bark beetle infestations of pine trees comprising contacting one or more fungal cells with an anti-fungal small molecule in an amount effective to reduce or inhibit conidial germination and mycelial growth,

wherein the anti-fungal small molecule is 5-(p-Bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid, and wherein the species of the fungal cell is selected from a group that has an obligate symbiosis with the mountain pine beetle (*Dendroctonus ponderosae*) and the western pine beetle (*Dendroctonus bervicomis*).

* * * * *